(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 July 2001 (12.07.2001)

PCT

(10) International Publication Number
WO 01/49204 A1

(51) International Patent Classification[7]: A61D 19/00, A61B 17/425

(21) International Application Number: PCT/AU01/00002

(22) International Filing Date: 3 January 2001 (03.01.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PQ 4998 7 January 2000 (07.01.2000) AU

(71) Applicant *(for all designated States except US)*: **BRESAGEN LIMITED** [AU/AU]; 38-39 Winwood Street, Thebarton, S.A. 5031 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GRUPEN, Christopher, Gerald** [AU/AU]; 59 Angas Road, Westbourne Park, S.A. 5041 (AU). **NOTTLE, Mark, Brenton** [AU/AU]; Lot P Alexander Avenue, RSD Bibaringa, S.A. 5118 (AU).

(74) Agents: **STEARNE, Peter, Andrew** et al.; Davies Collison Cave, Level 10, 10 Barrack Street, Sydney, NSW 2000 (AU).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/49204 A1

(54) Title: POLYSPERMIC FERTILIZATION IN *IN VITRO* FERTILIZATION SYSTEMS

(57) Abstract: This invention is directed to methods of animal production. The methods involve a reduction of polyspermic fertilization *in vitro* which includes incubating oocytes with sperm for a truncated time period so as to give oocytes with zona bound sperm, separating unbound sperm from the oocytes with zona bound sperm, and thereafter culturing the oocytes with zona bound sperm to allow fertilization to occur, and development to a desired development stage. The methods result in unbound sperm being removed by transferring oocytes with zona bound sperm to an insemination media free of sperm and allowing fertilization to proceed.

Polyspermic fertilization in *in vitro* fertilization systems

Field of Invention

This invention relates to improved methods of animal production. More particularly, to methods of reducing polyspermic fertilization during in vitro fertilization.

Background to the Invention

Polyspermic fertilization is rarely observed in nature. Two mechanisms control the number of sperm that fertilize the oocyte *in vivo*. The utero-tubal junction controls the number of capacitated sperm that enter the oviduct to fertilize the oocyte. In the pig this results in around 100 sperm being present at the site of fertilization in the oviduct.

Once sperm reach the site of fertilization, the first sperm to contact and penetrate the egg sets up a series of reactions within the oocyte resulting in the migration of cortical granules to the surface of the egg. These then release their contents (cortical granule reaction) which change the properties of the plasma membrane preventing further sperm from penetrating and fertilizing the egg, thus blocking polyspermy.

Polyspermy can be artificially induced, for example, in the pig, by increasing the number of sperm at the site of fertilization (Hunter R H F, (1996) *Molecular Reproduction and Development* 44:417-422). This suggests that the number of sperm at the site of fertilization is critical in determining the incidence of polspermy.

In current *in vitro* fertilization procedures oocytes are incubated with large numbers of sperm for about six hours or more, this time period representing the minimum period believed necessary for sperm penetration of oocytes. At the conclusion of this period the fertilized oocytes are washed to remove unbound sperm and incubated in culture medium to the appropriate developmental stage prior to storage, manipulation or embryo transfer. Polyspermic fertilization is a feature of current *in vitro* fertilization systems. For example, in the pig polyspermic fertilization has been shown to occur in 50% of oocytes matured and fertilized *in vitro* (reviewed by Funahashi H and Day B N, (1997)

Reproduction and Fertility Suppl. 52:271-283) .

The reasons for the high incidence of polyspermic fertilization *in vitro*, for example, in the pig, are believed to be due to:

- the use of much larger numbers of sperm to fertilize oocytes compared with that *in vivo*
- incomplete cytoplasmic maturation following *in vitro* maturation resulting in, amongst other things, a reduced cortical granule reaction which in turn reduces the block to polyspermy

Polyspermic fertilized eggs show limited development *in vitro* and do not generally develop to term *in vivo*.

A range of treatments have been examined in an attempt to reduce the incidence of polyspermy (reviewed by Funahashi H and Day B N, (1997) *Reproduction and Fertility Suppl.* 52:271-283). Reducing the number of sperm used for insemination for example can reduce the incidence of polyspermy but this also reduces overall fertilization rates. Other methods which have been examined include preincubation with follicular shells, use of follicular fluid etc. However none have proved entirely satisfactory for overcoming what is a major problem, for example, in porcine IVM/IVF systems.

The present invention provides a method which minimises the incidence of polyspermic fertilization, resulting in increased embryo development.

Summary of the Invention

In accordance with one aspect of the invention there is provided a method for reducing polyspermic fertilization *in vitro* which includes incubating oocytes with sperm for a truncated time period so as to give oocytes with zona bound sperm, separating unbound sperm from the oocytes with zona bound sperm, and thereafter culturing the oocytes with zona bound sperm to allow fertilization to occur, and development to a desired development stage.

The opportunity for additional sperm penetrating the egg may be minimised by limiting exposure of eggs to sperm. This may be achieved by transferring the oocytes with zona bound sperm, free from unbound sperm, to an insemination media and allowing fertilization to proceed, followed by culture in an embryo culture medium to a desired embryo development stage.

The truncated time period is a short time period, for example, from five to sixty minutes.

In other aspects the invention relates to an embryo produced by said methods and animals produced from such embryos.

Other embodiments of the invention are described hereafter.

Utilizing the method herein discussed, the inventors have shown that the development of *in vitro* matured oocytes to the blastocyst stage following insemination can be increased three-fold.

Detailed Description of the Invention

The method is applicable to all animal species including birds, fish, pigs, horses, cattle, sheep, goats, dogs, cats, and humans. Oocytes can be derived either *in vivo* or following *in vitro* maturation of immature oocytes obtained form slaughterhouse ovaries. *In vivo* derived oocytes can be derived by follicular aspiration *in situ* with or without hormonal stimulation. Sperm can be obtained from a variety of sources including sperm from fresh ejaculated, frozen or epididymal origin sperm. Embryos generated with this method can be used for a variety of basic and applied purposes. The latter includes embryo transfer, cryopreservation and nuclear transfer.

The present invention provides a method for reducing the incidence of polyspermy without compromising fertilization rates. Using this method the inventors have found that development is increased three fold.

The sperm used for fertilization may be provided in the same fashion as sperm currently used for *in vitro* fertilization. Thus, there is no reduction in sperm number used for fertilization. The fertilizing sperm may be an enriched sperm population where the highest motility and/or least aberrantly morphologically sperm are used.

Unbound sperm is separated from the oocytes following zona bound formation after the truncated time period. One convenient way of achieving this is to transfer oocytes with zona bound sperm to an insemination droplet containing no sperm. Alternatively, oocytes with zona bound sperm may be separated from non-bound sperm according to other standard procedures such as by transfer to media containing no sperm, or passing a flux of media over the oocyte with zona bound sperm so as to separate the oocyte from unbound sperm.

Oocytes are cultured in a suitable media well known in the art to a desired development stage. For example, the oocyte with zona bound sperm may be cultured to the stage of a two cell morula, four stage morula, eight stage morula, sixteen stage morula, or the blastocyst stage. The duration of culture depends upon the intended uses of the fertilized embryo, such as embryo transfer, cryopreservation, or nuclear transfer.

A non-limiting embodiment of the present invention will now be described with reference to porcine *in vitro* fertilization. It is to be understood the invention is in no way limited to the example provided. For convenience, the method of the present invention may be referred to as "two step insemination" or "two-step method".

Example

Oocytes recovered from the ovaries of slaughtered prepubertal gilts were matured in modified Medium 199 (25 oocytes/50 µl droplet) for forty six hours and sperm from fresh boar semen was prepared as described previously (Grupen C G *et al.*, (1999) *Reprod. Fertility and Development*, 9:571-575). Oocytes were denuded of cumulus cells by treatment with 1 mg/ml hyaluronidase and pipetting, and transferred to droplets (100 µl) of

modified TALP-PVA medium supplemented with 2.0 mM caffeine covered with mineral oil. Oocytes were then inseminated using either the standard or two-step method.

The standard(control) procedure involved incubating the gametes together in the one droplet for five hours. In the two-step method, oocytes were exposed to the same concentration of sperm as control oocytes (0.5 to 1.0 × 10^5 sperm/ml) for ten minutes in one droplet, and then transferred with the zona bound sperm to a second droplet (containing no sperm) for a five hour incubation. Following each procedure, oocytes were washed and transferred to droplets (50 µl) of NCSU-23 medium supplemented with 0.4% BSA covered with mineral oil. Putative zygotes were cultured at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Some zygotes were fixed sixteen hours later to assess penetration, while the remaining zygotes were cultured for seven days to assess development. The combined results of five replicates are shown in Table 1.

Table 1: Effects of the 2-step IVF procedure on sperm penetration and embryo development

	Control	Two-step
Oocytes examined after insemination:	113	107
Matured (% total ± SEM)	93.0 ± 3.1	93.9 ± 2.7
Fertilized (% matured ± SEM)	57.2 ± 19.8	79.9 ± 8.5*
Sperm/oocyte (mean ± SEM)	4.39 ± 0.39	2.84 ± 0.21*
Embryos examined after IVC:	153	151
Cleaved by 48 h (% total ± SEM)	69.5 ± 11.5	83.0 ± 10.2*
Day 7 blastocysts (% total ± SEM)	8.4 ± 3.5	30.0 ± 8.3*

*Value is significantly different to the corresponding control value ($P < 0.05$).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within its spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Claims

1 A method for reducing polyspermic fertilization *in vitro* which includes incubating oocytes with sperm for a truncated time period so as to give oocytes with zona bound sperm, separating unbound sperm from the oocytes with zona bound sperm, and thereafter culturing the oocytes with zona bound sperm to allow fertilization to occur, and development to a desired development stage.

2 A method according to claim 1 wherein unbound sperm is removed by transferring oocytes with zona bound sperm to an insemination media free of sperm and allowing fertilization to proceed.

3 A fertilized embryo when produced according to claim 1 or 2.

4 An animal produced from an embryo according to claim 3.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00002

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61D 19/00, A61B 17/425

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DWPI and PubMed + keywords

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Furnahashi H and Day B N "Advances in *in vitro* production of pig embryos", *Journal of Reproduction and Fertility Supplement* (1997) 52, 271-283	

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search
8 March 2001

Date of mailing of the international search report
16 March 2001

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pct@ipaustralia.gov.au
Facsimile No. (02) 6285 3929

Authorized officer

A.R. HENDRICKSON

Telephone No : (02) 6283 2415